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Expression Immunoassay. Antigen Quantitation Using Antibodies Labeled with Enzyme-Coding DNA Fragments

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A novel immunoassay is reported which uses an enzyme-coding DNA fragment as label (expression immunoassay). The DNA label is determined with high sensitivity by measuring the enzymatic activity produced after expression. A DNA fragment encoding the firefly luciferase is biotinylated and complexed with streptavidin. Biotinylated, specific antibodies are used for quantitation of antigen immobilized on microtiter wells. After completion of the immunoreaction, streptavidin–DNA is bound to the immunocomplex. Subsequent expression of the solid phase-bound DNA, by an one-step (coupled) cell-free transcription/translation, produces luciferase. The enzyme catalyzes the luminescent reaction of luciferin with O_2 and ATP. As few as 3000 molecules of DNA label can be detected. Also, 50 000 antigen molecules can be detected, and the luminescence is a linear function of the number of antigen molecules in a range extending over 3 orders of magnitude. The high sensitivity achieved is a result of the combined amplification due to transcription/translation and the substrate turnover.

Immunoassay is a powerful analytical technique used widely both in the investigation of the fine structure and function of biological systems and in the clinical laboratory for diagnosis and monitoring of various diseases.[1] In recent years, significant advances have emerged in immunoassay reagents (antibodies) and detection systems. Progress in antibody engineering has allowed the production of antibodies endowed with novel properties, such as bispecific and catalytic antibodies.[2,3] In parallel, considerable efforts have been focused on the improvement of immunoassay sensitivity.[1,4–6] Highly sensitive immunoassays are expected to provide valuable information on antigens found at levels that are close or below the current assay detection limit (e.g., antigens involved in early steps of signal transduction pathways), allow the monitoring of tumor-specific products in blood (for early detection of relapse), and facilitate the search for new diagnostic markers of disease. Sensitivity is determined mainly by the detectability of the molecules used for antibody labeling. Radioactive labels dominated in the immunoassay field for at least 2 decades. However, the current trend is toward novel nonisotopic systems with superior sensitivities. Nonisotopic immunoassays based on fluorescent, chemiluminescent, or enzyme labels have been developed and are commercially available.[1,4–6] Enzymes are probably the most widely used nonisotopic labels because they provide amplification through the high turnover of substrate to detectable products. The replacement of conventional chromogenic substrates with new ones that allow monitoring of enzymatic activity by fluorescence or chemiluminescence has further improved the sensitivity.[4–7]

Cell-free transcription and translation have been used extensively in the study of the factors involved in the regulation of gene expression.[8] In this report, the combined transcription/translation process is realized as a highly sensitive analytical system. Indeed, transcription entails synthesis of several mRNA molecules from each DNA template. Translation, in turn, produces more than one protein molecule from each transcript. If the DNA template encodes an enzymatically active protein, then amplification is further enhanced due to substrate turnover.

In expression immunoassay, the label is a DNA fragment that encodes an enzyme. The firefly luciferase-coding DNA is chosen as a model. The immunoreaction is carried out with biotinylated antibodies which, after the immunoreaction is completed, are attached to the DNA using streptavidin as a "bridge" molecule. The DNA is then subjected to coupled cell-free transcription/translation, which generates several luciferase molecules. Luciferase catalyzes the reaction of luciferin, ATP, and O_2 to produce oxyluciferin, AMP, pyrophosphate, CO_2, and light.[9,10] The luminescence is proportional to the number of antigen molecules present.

EXPERIMENTAL SECTION

Instrumentation. Luminescence measurements were carried out using a liquid scintillation counter (Model LS-6500, Beckman Instruments Inc., Fullerton, CA) in the single-photon monitoring mode. An imaging densitometer (Model GS-670, Bio-Rad Laboratories Ltd., Mississauga, Canada), along with the Molecular Analyst version 1.0 software, was used for quantitation of DNA fragments after agarose gel electrophoresis. The miniature horizontal gel system MLB-06 from Tyler Research Corp. (Edmonton, Canada) was used for electrophoresis. Time-resolved

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fluorescence was measured with the CFI 615 Immunoanalyzer from CyberFluor Division, Nordion International (Toronto, Canada). Excitation and emission wavelengths were set at 337 and 615 nm, respectively. High-performance liquid chromatography (HPLC) was performed using the Shimadzu system (Shimadzu Corp., Kyoto, Japan) with absorbance monitoring. The G24 environmental incubator shaker from New Brunswick Scientific (Edison, NJ) was employed for culturing bacteria.

Materials. For preparation of the DNA template, we used a plasmid contained, as a control DNA, in the TNT T7 wheat germ extract, a transcription/translation system commercially available from Promega Corp. (Madison, WI). The Wizard maxipreps DNA purification system and beetle luciferin were also from Promega. The restriction enzymes *Alw*44 I and *Pvu*II, as well as the Geneclean DNA purification system, were purchased from BioCan Scientific (Mississauga, Canada). Ultrapure 2′-deoxyribonucleoside 5′-triphosphates, the Klenow fragment of the *Escherichia coli* DNA polymerase I, and coenzyme A (CoA) were from Pharmacia Biotech (Montreal, Canada). Linear DNA markers (λ-DNA digested with *Eco*RI and *Hind*III) containing fragments from 0.12 to 21.2 kbp, supercoiled DNA markers (sizes 2.07−16.2 kbp), streptavidin, magnesium carbonate pentahydrate, and tricine were from Sigma (St. Louis, MO). Firefly luciferase (from *Photinus pyralis*), alkaline phosphatase-labeled streptavidin, adenosine triphosphate (ATP), bovine serum albumin, and the "blocking reagent" (Catalog No. 1096 176) were obtained from Boehringer (Laval, Canada). Biotin-14-dCTP (biotin attached at the N^4 position of cytidine by a 14-atom linker) and "U"-bottom polystyrene microtiter wells (Nunc, Maxisorp) were obtained from Life Technologies (Burlington, Canada). White, flat-bottom polystyrene wells, Microlite 2, were from Dynatech Laboratories Inc. (Chantilly, VA). Monoclonal anti-thyrotropin antibody was from Medix Biochemica (Finland). Biotinylated goat anti-mouse antibody was from Jackson Immunoresearch Laboratories, Inc. (distributed by BioCan). The phosphate ester of fluorosalicylic acid (FSAP) was from CyberFluor. Microcon-30 microconcentrators were purchased from Amicon Inc. (Beverly, MA).

The blocking solution contained 1% blocking reagent in 0.1 mol/L maleate and 0.15 mol/L NaCl, pH 7.5. The wash solution consisted of 50 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl, and 0.1% (v/v) Tween-20. The phosphate-buffered saline (PBS) contained 10 mmol/L sodium phosphate, 1.8 mmol/L potassium phosphate, 0.14 mol/L NaCl, and 2.7 mmol/L KCl, pH 7.4. The Tris−EDTA (TE) buffer consisted of 10 mmol/L Tris and 1 mmol/L EDTA, pH 8.0. A wheat germ-based transcription/translation mixture (wheat germ TNT system from Promega) was prepared according to the manufacturer's instructions. The complete mixture consisted of wheat germ extract (containing ribosomes, tRNA, and other translation factors), T7 RNA polymerase, and amino acids in the appropriate buffer.

Preparation and Purification of the DNA Template. For growing *E. coli* JM 109 cells, preparation of competent cells, and transformation with the plasmid DNA, we followed standard procedures.[8] Transformed bacteria were grown overnight in LB broth (10 g/L tryptone, 5 g/L yeast extract, 0.17 mol/L NaCl, and 2 mmol/L NaOH) containing 0.1 g/L ampicillin. The plasmid DNA was purified from a 1 L bacterial culture with the Wizard maxipreps DNA purification system according to the manufacturer's instructions. The size of the plasmid was confirmed by agarose (0.7%) gel electrophoresis and ethidium bromide staining, using the supercoiled DNA markers. The plasmid concentration was determined from the absorbance at 260 nm. For preparation of the DNA template, 40 μg of plasmid DNA was first digested for 90 min at 37 °C with 240 units of *Alw*44I in 150 μL of digestion buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol). Subsequently, a fill-in reaction was initiated by adding 150 μL of a solution containing 80 μmol/L of each of dATP, dGTP, dTTP, and biotin-14-dCTP as well as 40 units of the Klenow fragment of the DNA polymerase I. After incubation for 10 min at room temperature, the reaction was terminated by heating the mixture at 70 °C for 5 min. Subsequently, 240 units of *Pvu*II was added, followed by a 90 min incubation at 37 °C. After digestion, the DNA fragments were separated by agarose gel (0.7%) electrophoresis and stained with ethidium bromide. The band corresponding to 2.1 kbp was excised, and the DNA was purified using the Geneclean purification system and recovered in water. To quantitate the purified DNA template, we performed another electrophoresis and staining, as above. A lane containing the linear DNA markers was also included for construction of a calibration curve. The gel was photographed under UV excitation using a Polaroid 665 film, and the negatives were scanned by the densitometer.

Preparation and Purification of Streptavidin−DNA Template Complex. The streptavidin−DNA template complex was prepared in a final volume of 80 μL containing 3.6 μg (2.6 pmol) of (biotinylated) DNA template and 5 μg (84 pmol) of streptavidin in TE buffer. After incubating for 30 min at room temperature, the complex (50 μL) was purified by HPLC using a size exclusion column (the Bio-Sil Sec 400-5, 300 mm × 7.8 mm from Bio-Rad Laboratories) isocratically. The mobile phase was 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, and 150 mM NaCl, pH 6.8. The flow rate was 0.5 mL/min. Absorbance was monitored at 260 nm. A 1 mL fraction, corresponding to the void volume peak, was collected. Next, 100 μL of blocking solution was added (as a carrier), and the mixture was concentrated down to about 60 μL by using Microcon-30 microconcentrators. A 3 μL aliquot of this preparation was electrophoresed, and the DNA concentration was determined by scanning densitometry as above (see Preparation and Purification of the DNA Template).

Luciferase Assay. The substrate solution for luciferase contained 20 mmol/L tricine, 1.1 mmol/L magnesium carbonate pentahydrate, 2.7 mmol/L $MgSO_4$, 0.1 mmol/L EDTA, 33 mmol/L dithiothreitol, 270 μmol/L CoA, 530 μmol/L ATP, and 470 μmol/L luciferin, pH 7.8.[11] For the luciferase assay, 10 μL of the sample was added to 50 μL of substrate solution in a microcentrifuge tube. The tube was placed in a glass scintillation vial, and the luminescence was measured for 1 min using the liquid scintillation counter in the single-photon monitoring mode.

Quantitation of Immobilized Antigen by Expression Immunoassay. Solutions with various analyte concentrations were prepared by diluting a monoclonal anti-thyrotropin antibody in coating buffer (0.1 mol/L carbonate buffer, pH 9.6). The analyte was immobilized by pipetting 25 μL of solution into "U" bottom polystyrene microtiter wells and incubating overnight at 4 °C. The wells were then washed once with wash solution, and the remaining binding sites were blocked for 90 min at room temperature with blocking solution. Afterward, the wells were washed once as above, and to each well was added 25 μL of 5

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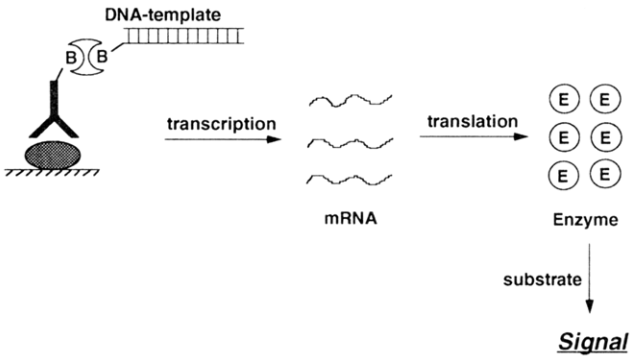

Figure 1. Principle of expression immunoassay. Antigen immobilized in microtiter wells is allowed to react with a biotinylated specific antibody. The immunocomplex is then reacted with streptavidin−DNA template. The solid phase-bound DNA is subjected to a coupled (one-step) transcription/ translation that produces enzyme molecules, which are subsequently detected by adding the appropriate substrate.

μg/mL biotinylated goat anti-mouse antibody, diluted in blocking solution. The immunoreaction was allowed to proceed for 30 min, and the wells were washed four times to remove the excess biotinylated antibody. Next, 25 μL/well of the streptavidin−DNA template complex (0.65 μg/mL with respect to DNA), diluted in blocking solution, was added. The wells were incubated for 10 min to allow for binding of the complex to biotinylated antibody, and the excess complex was removed by washing five times with wash solution and three times with TE buffer. Subsequently, 25 μL of the transcription/translation mixture was added into each well and incubated at 30 °C for 90 min, to allow expression of the DNA template bound to the immunocomplexes. At the end of this period, the synthesized luciferase was measured by adding 10 μL of the reaction mixture to 50 μL of substrate solution (as above).

Enzyme-Amplified, Time-Resolved Fluorescence Immunoassay of Immobilized Antigen. The reactions involved in enzyme-amplified, time-resolved fluorescence immunoassay, up to the addition of biotinylated antibody, were as described above (see Quantitation of Immobilized Antigen by Expression Immunoassay). Next, 25 μL of a solution containing 400 units/L streptavidin−alkaline phosphatase, 6% bovine serum albumin, 50 mmol/L Tris, and 0.5 g/L NaN_3, pH 8.0 were added and incubated for 15 min. The wells were washed four times with wash solution, and 25 uL/well of substrate solution (1 mmol/L fluorosalicyl phosphate, 0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 1 mmol/L $MgCl_2$, pH 9.1) was added. After a 30 min incubation at room temperature, the solution was transferred into white, flat-bottom microtiter wells, and 75 uL/well of a 0.4 mol/L NaOH, 2 mmol/L Tb^{3+}, 3 mmol/L EDTA, and 1 mol/L Tris, pH 12.5, solution was added. The wells were shaken for 1 min, and the fluorescence was measured with a time-resolved fluorometer.

RESULTS AND DISCUSSION

The principle of expression immunoassay is presented in Figure 1. The DNA template that was used as label was a linear DNA fragment prepared from a suitable plasmid (4.3 kbp) containing the luciferase-coding sequence downstream of a T7 RNA polymerase promoter (Figure 2a). The plasmid was first digested with *Alw*44I, a reaction that produced three fragments. The recessed 3′ ends created by *Alw*44I were filled-in with the Klenow fragment of DNA polymerase I in the presence of dATP, dGTP, dTTP, and biotin-dCTP. At the end of this step, both termini of each DNA fragment were biotinylated. Subsequent digestion with *Pvu*II removed a 0.49 kbp part from the biggest fragment, just upstream from the T7 promoter, thus leaving a 2.1 kbp fragment labeled with biotin only at the one terminus (Figure 2b,c). The fragments were separated by agarose gel electrophoresis and stained with ethidium bromide. The 2.1 kbp band was excised, and the DNA was purified and used as a label (DNA template). The concentration of the DNA template was determined by scanning densitometry.

To test if the DNA template was biotinylated, we mixed 0.5 μg of the template with a 30-fold molar excess of streptavidin (diluted in TE buffer) and incubated the mixture for 30 min. Solutions of the DNA with and without streptavidin were then electrophoresed and stained. In the presence of streptavidin, the DNA mobility decreased, due to the formation of streptavidin−biotin−DNA complexes. In parallel, a non-biotinylated DNA template was prepared exactly as above but using dCTP instead of biotin−dCTP. The electrophoretic mobility of this fragment was (as expected) not affected by the presence of streptavidin. Biotinylated and non-biotinylated DNA templates showed identical mobilities in the absence of streptavidin.

To assess the performance of transcription/translation as an analytical system, various amounts of DNA template were subjected to a coupled transcription/translation reaction for 90 min at 30 °C, in a total volume of 25 μL. After completion of the reaction, 10 μL aliquots of the mixtures were added to 50 μL of luciferase substrate solution, and the luminescence was measured for 1 min. Figure 3 shows that the luminescence is linearly related to the number of DNA template molecules in the range of 3×10^3−8×10^6. The curvature observed at higher numbers of molecules is due to saturation of the liquid scintillation counter from the light produced. From the signals obtained at various DNA levels and a luciferase calibration curve, prepared by diluting commercially available purified luciferase in the transcription/ translation reaction mixture, it was estimated that 12−14 molecules of luciferase are synthesized from each DNA template molecule.

The time dependence of luciferase synthesis from immobilized DNA template was studied by binding the biotinylated template on streptavidin-coated wells, followed by coupled transcription/ translation. Wells were coated overnight with 25 μL of 1.4 μg/ mL streptavidin in PBS and then washed three times with wash solution and blocked for 30 min with blocking solution. DNA template (25 μL), also diluted in blocking solution, was incubated for 30 min with the solid phase, and the wells were washed three times as above and once with TE buffer. Next 25 μL of transcription/translation mixture was added. During incubation at 30 °C, 1 μL aliquots were removed, and the luciferase was measured. The results (Figure 4) show that luciferase synthesis reaches a plateau after 90 min. The plateau has also been observed in liquid phase transcription/translation[12] (DNA template in solution) and is probably due to inactivation of translation factors with prolonged incubation.

The DNA template was attached to biotinylated antibodies by using streptavidin as a linker. We first prepared a 1:1 complex of the biotinylated DNA with streptavidin by reacting the template with a 30-fold molar excess of streptavidin. The complex was

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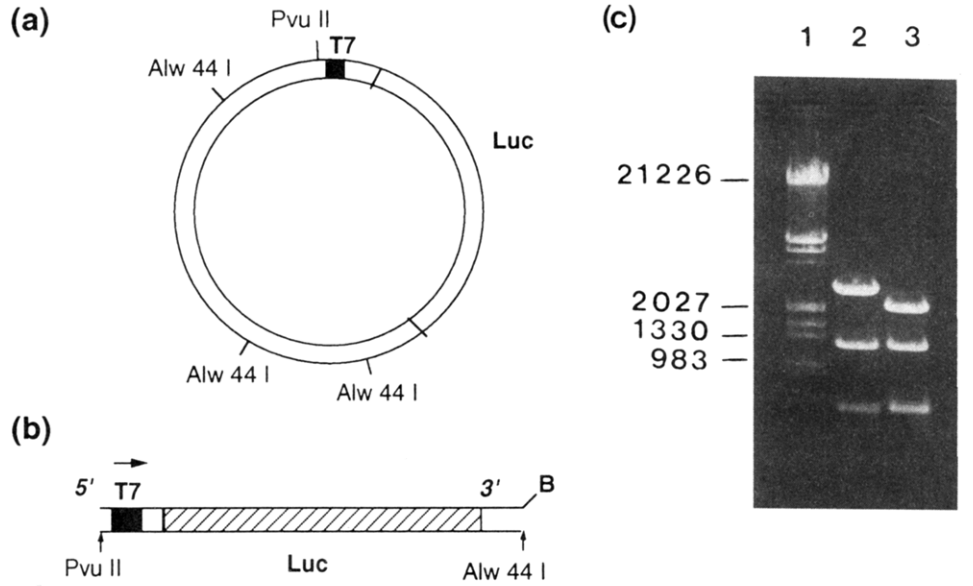

Figure 2. (a) Plasmid containing the T7 promoter and the luciferase-coding sequence (luc). (b) Structure of the DNA template. A single biotin molecule has been added downstream of the luciferase-coding sequence. (c) Analysis of the digested plasmid by 0.7% agarose gel electrophoresis and ethidium bromide staining. Lane 1, linear DNA markers (1.4 μg); lane 2, plasmid (1 μg) digested with *Alw*44I (fragment sizes: 2.59, 1.25, and 0.50 kbp); lane 3, plasmid (1 μg) after filling-in reaction and digestion with *Pvu*II (fragment sizes: 2.10, 1.25, and 0.50 kbp).

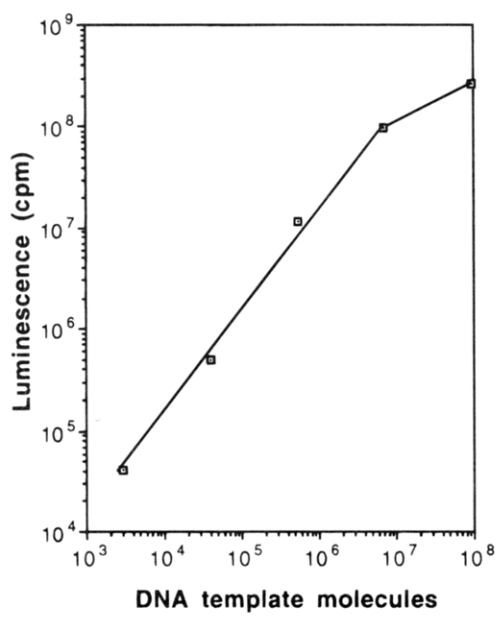

Figure 3. Quantitation of the luciferase-coding DNA template (label) by coupled transcription/translation.

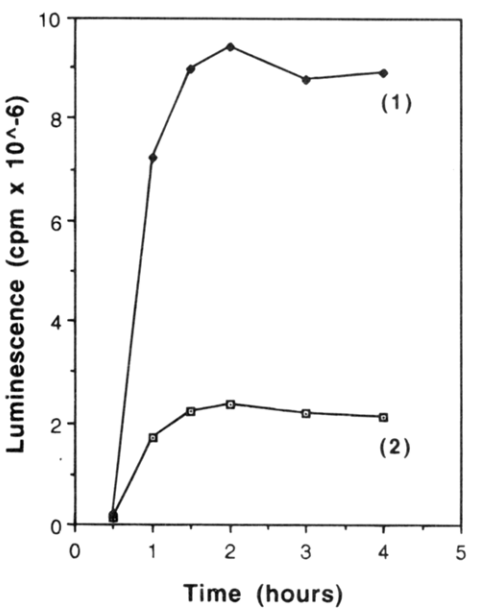

Figure 4. Time dependence of the expression of immobilized DNA template. Streptavidin-coated microtiter wells were incubated for 30 min with 25 μL of (1) 4.6 and (2) 0.9 ng/mL biotinylated DNA template solution. After washing, the immobilized template was expressed and luciferase activity monitored.

separated from free streptavidin by size-exclusion HPLC. The streptavidin–DNA template complex was eluted at the void volume (9.5 min), whereas free streptavidin came off at 20.3 min.

To assess the performance of expression immunoassay, we immobilized various amounts of antigen (an anti-thyrotropin monoclonal antibody) on microtiter wells and used a biotinylated goat anti-mouse antibody for detection. After completion of the immunoreaction, the streptavidin–DNA was bound to the immunocomplexes; the excess reagent was washed out and the transcription/translation mixture added directly to the solid phase. During the 90 min incubation period, DNA template molecules bound to the immunocomplexes were expressed, and luciferase was synthesized. Aliquots of the reaction mixtures were then mixed with substrate solution, and the luminescence was measured. The results are presented in Figure 5. There is a linear relationship between luminescence and the amount of antigen present in the well, in the range of 5×10^4–1×10^8 molecules. After antigen immobilization and blocking, the time required for detection was about 130 min. The CV obtained at the level of 10^6 molecules was 7.5%.

Expression immunoassay was compared directly with enzyme-amplified, time-resolved fluorometric immunoassay, one of the most sensitive immunoassay systems currently available.[13–15] In

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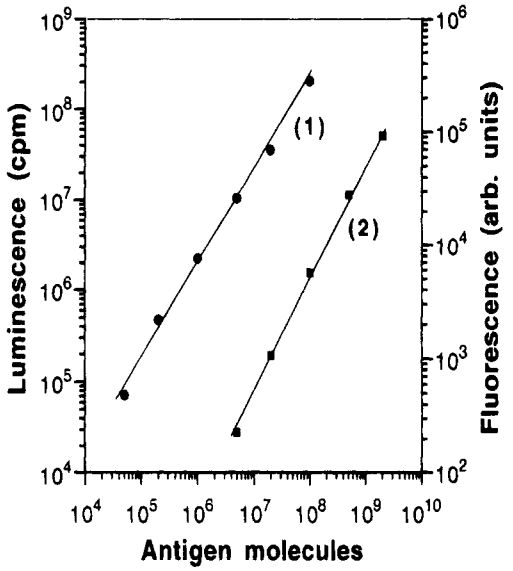

Figure 5. Quantitation of antigen immobilized on microtiter wells by expression immunoassay (1) and enzyme-amplified, time-resolved fluorescence immunoassay (2). The assays were performed as described in the Experimental Section. The luminescence and the time-resolved fluorescence measured were plotted against the number of molecules of antigen in the well.

this assay, after completion of the immunoreaction, alkaline phosphatase-labeled streptavidin was added into the wells. Subsequent hydrolysis of the substrate (FSAP) produced fluorosalicylate, which forms fluorescent complexes with Tb^{3+}–EDTA. The fluorescence was linearly related to the number of antigen molecules in the range of 5×10^6–1×10^9 (Figure 5).

For expression immunoassay to be feasible and highly sensitive, the following characteristics are desirable: (a) Posttranslational modification of the synthesized protein is not required for full enzymatic activity. This is because the current in vitro transcription/translation systems do not allow for specific modifications after expression. (b) The enzyme can be monitored directly and conveniently in the transcription/translation mixture. (c) Preferably, the enzyme should either consist of a single polypeptide chain or be an oligomer of identical subunits. In the case of oligomeric enzymes, however, the number of fully active enzyme molecules synthesized from each DNA template decreases as the number of subunits increases. (d) Short DNA templates (encoding low molecular weight enzymes), in principle, should give higher transcription/translation yields than longer ones and thus higher sensitivities. Luciferase was chosen as a model in this work because it combines the above characteristics. It is a single polypeptide chain (550 amino acids) and requires no posttranslational modification, and the luciferase cDNA has been used as a reporter gene in biological studies, where the enzymatic activity was measured conveniently in various tissue extracts.[9,10,16]

The use of coupled transcription/translation in the present work greatly enhances the practicality of the proposed system by eliminating the need for purification of the mRNA transcripts before translation.

Previous attempts at direct chemical conjugation of luciferase to antibodies have shown that the enzyme is inactivated during coupling reactions.[4] Progress in DNA technology has allowed the preparation of recombinant fusion proteins in which the enzyme is "genetically" conjugated to protein A,[17] a protein that binds to the Fc portion of immunoglobulin G. Although this approach gives better conjugates than chemical coupling, there is only one enzyme molecule attached per antibody. In contrast, by using an enzyme-coding DNA fragment as label, instead of the enzyme itself, problems of inactivation due to coupling are avoided, a significant amplification is achieved, and the generated enzyme molecules are free in the solution.

DNAs are generally more stable than proteins (enzymes). Nucleases (if present in the sample) are not expected to affect a noncompetitive immunoassay. The reason is that after antigen immobilization (or capture), the solid phase is washed several times. Similarly, a washing step follows the addition of biotinylated detection antibody. Therefore, all the sample constituents that could degrade the DNA label or interfere with the transcription/translation reactions are efficiently removed.

Recently a highly sensitive detection system, termed immuno-PCR (polymerase chain reaction), has been reported.[18] Although both systems use DNA as a label, the underlying concepts are quite different. Immuno-PCR replicates the label, thus generating a large number of copies which are then detected by electrophoresis. On the other hand, the system proposed here expresses the label into a protein with catalytic activity. As a consequence, the requirements for the labels differ considerably. Any DNA fragment can be used as a label for immuno-PCR along with suitable primers. The DNA label in expression immunoassay, however, is designed to contain sequences that enable it to function as a template for subsequent transcription and translation.

We have demonstrated the principle of expression immunoassay with a DNA template prepared from a commercially available vector without optimizing its structure. The sensitivity could be further enhanced by incorporating appropriate promoter, enhancer, or termination sequences in the DNA template that ensure the highest yield of the transcription/ translation step.[19,20] In principle, expression should produce hundreds of enzyme molecules per DNA template. These possibilities are currently being investigated in our laboratory.

In conclusion, the contribution of the present work lies in the fact that it introduces (for the first time) a nucleic acid fragment/complete expression unit as a label in immunoassays and demonstrates that the transcription/translation process can form the basis for a highly sensitive analytical system.

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